

ON THE UNCERTAINTY IN SINGLE MOLECULE FLUORESCENT  
LIFETIME AND ENERGY EMISSION MEASUREMENTS

TECHNICAL REPORT 94-03

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OCTOBER 1994

REVISED 04/06/95 4:04 PM

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(NASA-CR-198009) ON THE  
UNCERTAINTY IN SINGLE MOLECULE  
FLUORESCENT LIFETIME AND ENERGY  
EMISSION MEASUREMENTS  
(Massachusetts General Hospital)  
24 p

N95-24877

Unclass

G3/72 0045514

## **Abstract**

Time-correlated single photon counting has recently been combined with mode-locked picosecond pulsed excitation to measure the fluorescent lifetimes and energy emissions of single molecules in a flow stream. Maximum likelihood (ML) and least squares methods agree and are optimal when the number of detected photons is large however, in single molecule fluorescence experiments the number of detected photons can be less than 20, 67% of those can be noise and the detection time is restricted to 10 nanoseconds. Under the assumption that the photon signal and background noise are two independent inhomogeneous Poisson processes, we derive the exact joint arrival time probability density of the photons collected in a single counting experiment performed in the presence of background noise. The model obviates the need to bin experimental data for analysis, and makes it possible to analyze formally the effect of background noise on the photon detection experiment using both ML or Bayesian methods. For both methods we derive the joint and marginal probability densities of the fluorescent lifetime and fluorescent emission. The ML and Bayesian methods are compared in an analysis of simulated single molecule fluorescence experiments of Rhodamine 110 using different combinations of expected background noise and expected fluorescence emission. While both the ML or Bayesian procedures perform well for analyzing fluorescence emissions, the Bayesian methods provide more realistic measures of uncertainty in the fluorescent lifetimes. The Bayesian methods would be especially useful for measuring uncertainty in fluorescent lifetime estimates in current single molecule flow stream experiments where the expected fluorescence emission is low. Both the ML and Bayesian algorithms can be automated for applications in molecular biology.

## Introduction

In the last four years it has become possible to record temporal data from individual fluorescent dye molecules in flow streams and to estimate single molecule fluorescent lifetime and energy emissions (21). Perfection of this technology should significantly enhance the analytic sensitivity of fluoroimmunoassays, capillary zone electrophoresis, flow cytometry, DNA fingerprinting, fragment sizing sequencing. Proper application of the single molecule fluorescence techniques in these detection systems requires accurate assessment of the uncertainty in the measurement of the molecule's lifetime and energy emission. As a consequence, there is much interest in characterizing the statistical properties of photon data collected in single molecule excitation experiments (19).

Hall and Selinger (5) studied the general problem of decay lifetime estimation and derived equations for the maximum likelihood (ML), method of moments and least squares (LS) estimations of the parameters in an inhomogeneous Poisson process model of photon detection in the absence of background noise. They also reported large sample formulae for the variances of these estimators, and showed that the ML estimation procedure was the most statistically efficient and that it was also mathematically tractable. Peck et al. studied single molecule fluorescence detection with the signal and background noise processes modelled as two independent homogeneous Poisson processes and described an autocorrelation procedure for burst detection. Tellinghuisen and Wilkerson studied the performance of ML and LS methods for estimating fluorescent lifetimes using an arrival time model in the case where  $N$ , the numbers of detected photons, is small and  $T$ , the observation time, is finite. They found that both estimates were biased and that the variance of the ML estimate was formally divergent. They suggest that the  $(N/N-1)$  bias in the reciprocal of the lifetime parameter could be easily corrected and that statistical models parameterized in terms of this parameter were more analytically tractable. Tellinghuisen studied the properties of least squares (LS)

techniques for estimating fluorescent lifetimes in the presence of background noise using Monte Carlo methods and partial derivative matrices to perform error analyses. He reported that background noise reduced the precision in the lifetime estimate by an order of magnitude and that this measurement precision could be recovered by incorporating into the analysis a model of the background noise based on the properties of the detection system studied in the absence of a fluorescent experiment.

In the study of photon bursts from single Rhodamine 110 dye molecules dissolved in methanol, Tellinghuisen et al. compared the use of an approximate arrival time plus background noise model analyzed with LS methods to an arrival time noise free model analyzed by ML procedures. In their LS analysis the error in the reciprocal of the lifetime parameter exceeded the  $N^{-1/2}$  expected for data collected over an infinite time interval in the absence of background noise. Because the model analyzed with the LS methods included background noise and because problems in the parameter estimation could be readily diagnosed with contour plots of the minimum  $\chi^2$  function, these authors concluded that the LS approach offered the preferred means of analyzing the effect of background noise in single molecule fluorescence experiments.

The principal analytic issues to be addressed are: (1) proper specification of a probability model for photon detection from single molecules in the presence of background noise; and (2) formulation of a statistical estimation procedure based on that model which allows accurate determination of single molecule fluorescent emissions and lifetime when emissions are low background noise is significant and the detection interval is finite. There is significant evidence to suggest that detection of photon from single fluorescent molecule may be modelled a Poisson process. Therefore, under the assumption that the signal and background noise are two independent inhomogeneous Poisson processes, we derive the exact joint probability density of photon arrivals in a finite time interval from a single fluorescent molecule derive both ML and Bayesian procedures for estimating the fluorescence lifetime energy emission and their associated

uncertainties in terms of well defined probability densities. We compare the ML and Bayesian methods in an analysis of simulated data from Rhodamine 110 for various combinations of expected fluorescence emissions and background noise.

### Model Derivation

We assume that the arrival times of the photons from a single fluorescent molecule are recorded in a finite interval  $[0, T]$ . Let  $t_i$  be the arrival time of the  $i^{\text{th}}$  photon, where  $0 < t_1, \dots, < t_I < T$ . We assume that the number of photons detected from the fluorescent species is a Poisson process with continuous intensity function  $\lambda(t) = Ae^{-t/\tau}$ , for  $t \geq 0$ . The parameter  $\tau$  is the average fluorescent lifetime and  $A$  is the number of photons detected at time 0. Let  $\Lambda(t) = \int_0^t \lambda(u) du$ . The quantity  $\Lambda(T)$  is the average fluorescence emission in the observation interval  $(0, T]$ . We assume that the number of background photons detected is a Poisson process with a continuous intensity function  $q(t)$ ,  $t \geq 0$ . Forms of  $q(t)$  which have been reported include the constant function and a function composed of a linear combination of two Gaussian functions, and exponential function and a constant. Let  $Q(t) = \int_0^t q(u) du$ . We assume that  $q(t)$  is known from background photon measurements made on the detection system in the absence of the fluorescent molecules. We assume also that

$$\lim_{b \rightarrow \infty} \int_0^b q(u) du = \infty \quad (1)$$

Equation (1) is a technical assumption which ensures that the joint, conditional and marginal probability densities of the arrival times are well defined. Under the assumption that the fluorescence emission and background noise processes are independent, the number of photons arriving from both is a Poisson process with intensity function  $\lambda(t) + q(t)$ .

To derive the joint probability density of photon arrival times we consider the event that  $I$  photons arrive in  $(0, T]$  at times  $0 < t_1 < t_2, \dots, < t_I \leq T$ . This probability density is defined by the events that no arrivals occur in the intervals  $(0, t_1], (t_i + \Delta t_i, t_{i+1}]$  for  $i = 1, \dots, I-1$  and  $(t_I + \Delta t_I, T]$ , and that exactly one arrival occurs in each of the intervals  $(t_i, t_i + \Delta t_i]$  for  $i = 1, \dots, I$ . By the definition of an inhomogeneous a Poisson process the following statements describe the probabilities of these events:

$$\begin{aligned} \Pr(\text{No arrival in } (0, t_1]) &= \exp\left\{-\int_0^{t_1} \lambda(u) + q(u) du\right\} \\ \Pr(\text{No arrival in } (t_i + \Delta t_i, t_{i+1}]) &= \exp\left\{-\int_{t_i + \Delta t_i}^{t_{i+1}} \lambda(u) + q(u) du\right\} \\ \Pr(\text{One arrival in } (t_i, t_i + \Delta t_i]) &= \int_{t_i}^{t_i + \Delta t_i} \lambda(u) + q(u) du \exp\left\{-\int_{t_i}^{t_i + \Delta t_i} \lambda(u) + q(u) du\right\} \end{aligned} \quad (2)$$

The intervals are nonoverlapping and thus, independent by the basic axioms of a Poisson process. The joint probability of the events is therefore,

$$\begin{aligned} f(t|\tau, A) \prod_{i=1}^I \Delta t_i &= \exp\left\{-\int_0^{t_1} \lambda(u) + q(u) du\right\} \prod_{i=1}^{I-1} \exp\left\{-\int_{t_i + \Delta t_i}^{t_{i+1}} \lambda(u) + q(u) du\right\} \exp\left\{-\int_{t_I + \Delta t_I}^T \lambda(u) + q(u) du\right\} \\ &\times \prod_{i=1}^I \int_{t_i}^{t_i + \Delta t_i} \lambda(u) + q(u) du \exp\left\{-\int_{t_i}^{t_i + \Delta t_i} \lambda(u) + q(u) du\right\} + o\left(\prod_{i=1}^I \Delta t_i\right) \end{aligned} \quad (3)$$

where  $t = (t_1, t_2, \dots, t_I)^T$ . Dividing both sides by  $\prod_{i=1}^I \Delta t_i$  and letting  $\Delta t_i \rightarrow 0$  for each  $i$  gives the joint probability density of the arrival times

$$\begin{aligned} f(t|\tau, A) &= \prod_{i=1}^I [\lambda(t_i) + q(t_i)] \exp\left\{-\int_0^{t_1} \lambda(u) + q(u) du\right\} \exp\left\{-\int_{t_I}^T \lambda(u) + q(u) du\right\} \\ &\times \prod_{i=1}^{I-1} \exp\left\{-\int_{t_i}^{t_{i+1}} \lambda(u) + q(u) du\right\} \end{aligned} \quad (4a)$$

$$= \prod_{i=1}^I [\lambda(t_i) + q(t_i)] \exp\left\{-\int_0^T \lambda(u) + q(u) du\right\} \quad (4b)$$

for  $0 < t_1 < \dots < t_I < T$ , where we used the fact that

$$\frac{\int_{t_i}^{t_i + \Delta t_i} \lambda(u) + q(u) du}{\Delta t_i} \rightarrow \lambda(t_i) + q(t_i) \quad (5)$$

as  $\Delta t_i \rightarrow 0$ , for all  $i$ .

Equation (4) shows that the joint probability density of the photon arrivals may be represented without the need to bin the data. This derivation may be viewed as taking the number of bins equal to the number of detected photons, placing the  $i^{th}$  photon arrival in a bin of width  $\Delta t_i$ , and letting the widths of all bins go to zero. From equation (4b) it appears that the  $t_i$ 's are a collection of independent observations on the interval  $(0, T]$ , however, they are not. Their joint probability density is defined on  $r^i$  subject to the constraint that  $0 < t_1 < t_2, \dots, < t_I < T < \infty$ . In particular, equation (4a) shows that the arrival times are a Markov process in that the conditional probability density of  $t_i$  depends only on  $t_{i-1}$ . That is, we may write

$$f(t|\tau, A) = \prod_{i=1}^I f(t_i|t_{i-1})$$

where  $f(t_i|t_{i-1}) = (\lambda(t_i) + q(t_i)) \exp\left\{-\int_{t_{i-1}}^{t_i} \lambda(u) + q(u) du\right\}$  and  $t_i$  is defined on the interval  $(t_{i-1}, \infty)$ . The practical reason for observing that the arrival times obey a Markov process is that this representation of the joint arrival time density suggests a simple method of

simulating photon arrival times for an inhomogeneous Poisson signal plus noise model (Lewis and Schedler, 1977). This algorithm is presented in the **Appendix**.

### Conditional Arrival Time Probability Density

The observed photon arrival time data may also be analyzed in terms of the conditional arrival time probability density. That is, given  $I$  photon arrivals detected in  $(0, T]$ , what are the probable locations of the arrivals in that interval? The number of photons arriving in  $(0, T]$ ,  $N(T)$ , is distributed as a Poisson random variable with parameter  $\Lambda(T)$ . From equation (4) and the properties of a Poisson process the conditional arrival time probability density is

$$f(t|T, A, I) = \Pr\{0 < t_1 < \dots < t_I \leq T \text{ and } N(T) = I\} / \Pr\{N(T) = I\}$$

$$= \frac{\prod_{i=1}^I [\lambda(t_i) + q(t_i)] \exp\{-\int_0^T \lambda(u) + q(u) du\}}{[\Lambda(T) + Q(T)]^I \exp\{-[\Lambda(T) + Q(T)]\} / I!} \quad (6)$$

$$= \frac{I! \prod_{i=1}^I [\lambda(t_i) + q(t_i)]}{[\Lambda(T) + Q(T)]^I}$$

In the case where the Poisson intensity parameter is constant and the observation interval is divided into  $k$  bins, equation (6) is a  $k$ -nominal probability mass function.

### Statistical Analysis of Experimental Data

Maximum Likelihood Approximation of the Fluorescent Lifetime and Energy Emission Probability Densities.



From the joint arrival time density we can estimate  $\tau$  and  $A$  by the method maximum likelihood. The log likelihood based on the joint arrival time density is

$$\log f(t|\tau, A) = \sum_{i=1}^I \log(\lambda(t_i) + q(t_i)) - [\Lambda(T) + Q(T)] \quad (7)$$

and the ML estimates can be obtained numerically by finding the values of  $\tau$  and  $A$  which maximize Equation (7). Similarly, the log likelihood for the conditional arrival time density in equation (6) is

$$\begin{aligned} \log f(t|\tau, A, I) &= \sum_{i=1}^I \log(\lambda(t_i) + q(t_i)) - I \log[\Lambda(T) + Q(T)] \\ &\quad + \log(I!). \end{aligned} \quad (8)$$

Equation (8) may also be used for ML estimation of  $\tau$  and  $A$ .

Under the assumption that  $A$  is large we can derive approximate probability densities for  $\tau$  and  $A$ . Let  $\mu_x = (\hat{\tau}, \hat{A})^T$  be the ML estimates of  $\tau$  and  $A$  respectively and define  $x = (\tau, A)$ . The observed information matrix  $G$  is the 2x2 matrix whose elements are  $G_{11} = \frac{\partial^2 \log f(t|\tau, A)}{\partial \tau^2}$ ,  $G_{12} = G_{21} = \frac{\partial^2 \log f(t|\tau, A)}{\partial \tau \partial A}$  and  $G_{22} = \frac{\partial^2 \log f(t|\tau, A)}{\partial A^2}$ . It follows from the large sample theory of ML estimates that the approximate joint probability density of  $\tau$  and  $A$  is the Gaussian density defined as

$$\hat{f}_1(\tau, A|t) = \frac{1}{2\pi|V|^{\frac{1}{2}}} \exp\left\{-\frac{1}{2}(x - \mu)^T V^{-1}(x - \mu)\right\} \quad (9)$$

where  $V = -G^{-1}$  and  $|V|$  is the determinant of  $V$ . The marginal probability density of  $\hat{f}(\tau|t)$ , is the Gaussian density with mean  $\hat{\tau}$  and variance equal to  $V_{11}$ , i.e. the 1,1 element

of  $\nu$ . To compute the approximate probability density of  $\Lambda(T)$ , we define the transformation

$$\begin{aligned} y &= h \begin{pmatrix} \tau \\ A \end{pmatrix} = \begin{pmatrix} \tau \\ \Lambda(T) \end{pmatrix} \\ \mu_y &= h \begin{pmatrix} \hat{\tau} \\ \hat{A} \end{pmatrix} \end{aligned} \quad (10)$$

and its Jacobian

$$J = \begin{bmatrix} 1 & 0 \\ A[1 - (1 + \frac{T}{\tau})e^{-T/\tau}] & \tau(1 - e^{-T/\tau}) \end{bmatrix}$$

The determinant of  $J$  is  $|J| = \tau(1 - e^{-T/\tau})$ . Applying the change of variables in equation (10) to  $\hat{f}_1(\tau, A|t)$  under the assumption that  $A$  is large, shows that the approximate joint probability density of  $\tau$  and  $\Lambda(T)$  is the Gaussian density defined as

$$\hat{f}_2(\tau, \Lambda(T)|t) = \frac{1}{2\pi|\Sigma|^{\frac{1}{2}}} \exp\{-\frac{1}{2}(y - \mu_y)^T \Sigma^{-1}(y - \mu_y)\} \quad (11)$$

where  $\Sigma = JJ^T$ . The approximate marginal probability density of  $\Lambda(T)$  is the Gaussian density with mean  $\hat{\Lambda}(T)$  and variance  $\Sigma_{22}$ .

Bayesian Estimation of the Fluorescent Lifetime and Energy Emission Probability Densities.

To conduct a Bayesian analysis of the lifetime and energy emission estimation problem we assume that knowledge about  $\tau$  and  $A$  known prior to the experiment can be summarized in terms of a prior probability density. Since the fluorescent lifetime is

independent of  $A$ , the number of photons observed at time 0, we write the joint prior density of  $\tau$  and  $A$  as the product of two locally uniform prior densities defined as  $f(\tau, A) = f(\tau)f(A)$  where,

$$f(\tau) = \begin{cases} (\tau_2 - \tau_1)^{-1} & \tau_1 \leq \tau \leq \tau_2 \\ 0 & \text{otherwise} \end{cases}$$

$$f(A) = \begin{cases} (A_2 - A_1)^{-1} & A_1 \leq A \leq A_2 \\ 0 & \text{otherwise} \end{cases} \quad (12)$$

The values of  $\tau_1, \tau_2, A_1$  and  $A_2$  are defined from known properties of the particular single molecule experiment. Applying Bayes theorem equations (4) and (12) yields the joint posterior density of

$$f_1(\tau, A|t) = \frac{f(\tau)f(A)f(t|\tau, A)}{f(t)} \quad (13)$$

where  $f(t) = \int \int_{\tau, A} f(\tau)f(A)f(t|\tau, A)dA d\tau$ .

Equation (12) shows that the locally uniform prior densities can be used to constrain the parameter values to a region in the  $\tau - A$  plane which is physically plausible. Equation (13) shows that the uncertainty in the parameter defined by the Bayesian analysis is the product of the ranges defined by the prior densities and the uncertainty in or information about the parameters derived from the experiment, and summarized in the likelihood function  $f(t|\tau, A)$ . That is, the Bayesian analysis combines prior information with that collected in the experiment to determine the uncertainty in the model parameters. Because the prior density is uniform on the  $\tau - A$  plane and because the likelihood and log likelihood are equivalent summaries of the experimental information,

if the experiment contains a lot of information about the model parameters then the uncertainty in the model parameters defined by the ML and Bayesian analyses will agree.

The posterior probability density of the fluorescent lifetime is obtained by integrating (13) with respect to  $A$  to yielding

$$f_1(\tau|\mathbf{t}) = \int_A f_1(\tau, A|\mathbf{t}) dA \quad (14)$$

Since we are interested in  $\Lambda(T)$  instead of  $A$  we apply the change of variables defined by equation (10) and find that the joint posterior probability density of the fluorescent lifetime and fluorescent emission is

$$f_2(\tau, \Lambda(T)|\mathbf{t}) = \frac{1}{|J|} f_1(\tau, \Lambda(T)\tau^{-1}(1 - e^{-T/\tau})|\mathbf{t}) \quad (15)$$

The marginal posterior density of  $\Lambda(T)$  is defined as

$$f_2(\Lambda(T)|\mathbf{t}) = \int f_2(\tau, \Lambda(T)|\mathbf{t}) d\tau \quad (16)$$

Equations (14) and (16) define the uncertainty in the fluorescent lifetime and energy emission respectively in terms of probability densities without having to make large sample assumptions. The respective modes, medians and means of  $f_1(\tau|\mathbf{t})$  and  $f_2(\Lambda(T)|\mathbf{t})$  may be used as point estimates of  $\tau$  and  $\Lambda(T)$ .

### **Simulation Study of Rhodamine 110 Single Molecule Fluorescence Detection**

We compared the ML and Bayesian methods in a study of simulated single molecule fluorescence experiments for Rhodamine 110 assuming an expected fluorescent

lifetime and detection interval of 4.2 and 10 nanoseconds respectively based on Tellinghuisen, et. al. We simulated three levels of expected background noise fraction: high = 0.70, moderate = 0.35 and none = 0 combined with four levels of expected fluorescence emission: low = 19.1, medium =191, moderate =952 and high =3812 photons 10 nanoseconds. These expected fluorescence emission levels are the values of  $\Lambda(T)$  obtained with  $A$  set respectively to 5, 50, 250 and 1000 with  $\tau=4.2$  and  $T=10$ . We assumed  $q(t) = \lambda_n$ , a constant function, whose value was determined for any given specification of the noise fraction and expected fluorescence by the relation

$$nf = \frac{\lambda_n T}{\Lambda(T) + \lambda_n T}$$

where  $nf$  is the expected background noise fraction and  $\lambda_n T = Q(T)$ .

For each combination of  $nf$  and  $\Lambda(T)$  we simulated 3 detection experiments (36 in total) using the algorithm defined in the **Appendix**. ML estimation was carried out by a combination quasi-Newton's method and local search procedure and the Bayesian methods by rectangular integration procedures. For the Bayesian analysis we took  $\tau_1 = 0$  and  $\tau_2 = 12$  nanoseconds in the prior density for  $f(\tau)$ . Given previous reports on the fluorescent lifetime of Rhodamine 110, it is reasonable to assume that  $\tau$  would lie in this interval. For  $f(A)$  we chose four different representations for both  $A_1$  and  $A_2$  depending on value of  $A$  used in the • simulations. These were

$A$	$A_1$	$A_2$
5	0	10
50	0	100
250	150	350
1000	750	1250

### *Maximum Likelihood Analysis*

As  $nf$  decreased and  $ef$  increased, the precision in both the fluorescent lifetime and energy emission estimates increased (Table 1). The CV's of the lifetime and energy emission estimates decreased respectively from 58.03% and 46.8% ( $nf = 0.35$  and  $ef = 19.1$ ) to 2.68% and 1.61% ( $nf = 0.0$  and  $ef = 3812$ ). In all the simulations the true values of  $\tau$  and  $\Lambda(T)$  are covered by the 0.95 confidence intervals based on the maximum likelihood estimates (mean  $\pm$  2SD). The widths of the 95% confidence intervals (4SD) for  $\tau$  ranged from 8.4 ns ( $nf = 0.35$  and  $ef = 19.1$ ) to 0.44 ns ( $nf = 0.0$  and  $ef = 3812$ ). The absolute widths of the 0.95 confidence intervals for  $\Lambda(T)$  increased with increasing values of  $\Lambda(T)$  however, the length of the interval as a percentage of  $\Lambda(T)$  decreased from 93.6% ( $nf = 0.35$  and  $ef = 19.1$ ) to 6.6% ( $nf = 0.0$  and  $ef = 3812$ ), i.e. twice the CV's. Due to their low signal to noise ratio the simulations with  $ef = 19.1$  gave point estimates of  $\tau$  which agreed least with the intended value of 4.2 ns. The increase in estimation precision of  $\tau$  and  $\Lambda(T)$  with decrease in  $nf$  and increase in  $ef$  is shown in Figure 1. The estimated joint density for  $nf = 0.35$  and  $ef = 19.1$  has approximately 10% of its support on negative values of  $\tau$  and  $\Lambda(T)$  because the Gaussian approximation based on the ML estimates is not restricted to positive values (Figure 1A). Across all the simulations the approximate joint densities of  $\hat{\tau}$  and  $\hat{\Lambda}(T)$  have slight positive correlations (0.31 to 0.35) for  $nf = 0.70$  (Figures 1A, D and G) whereas the two estimates were uncorrelated for  $nf = 0.0$  (Figures 1C, F and I).

### *Bayesian Analysis*

For the Bayesian analysis as  $nf$  decreased and  $ef$  increased, the precision in both the fluorescent lifetime and energy emission estimates also increased (Tables 2 and 3 and Figure 2). The CV's of the lifetime and energy emission estimates decreased respectively from 55.03% and 41.51% ( $nf = 0.35$  and  $ef = 19.1$ ) to 2.69% and 1.61% ( $nf = 0.0$  and  $ef =$

3812). In all the simulations the true values of  $\tau$  and  $\Lambda(T)$  are covered by the 0.95 credibility intervals, i.e. the Bayesian equivalent of the 0.95 confidence intervals. For  $ef = 952$  and 3812 there was very good agreement between the joint probability densities of  $\tau$  and  $\Lambda(T)$  and each of their marginal probability densities (Tables 1, 2 and 3). All the Bayesian probability densities for these values of  $ef$  appeared to be Gaussian as suggested by their plots and the fact that their skewness and kurtosis values are all close to zero. Similarly, for the simulations in which  $ef = 191$  and  $nf$  equal to 0.35 or 0.0, there was good agreement between the ML and Bayesian probability density estimates (Figures 1E and 2F, and Figures 2E and 2F).

For  $ef = 19.1$  for all values of  $nf$  and for  $ef = 191$  and  $nf = 0.70$  the Bayesian estimates of the joint probability densities of  $\tau$  and  $\Lambda(T)$  are non-Gaussian (Figures 2A, 2B, 2D and 2G). The marginal probability densities for  $\Lambda(T)$  are nearly Gaussian for these four combinations of  $ef$  and  $nf$  and these densities are in very good agreement with their ML counterparts. The case in which the marginal density is least Gaussian is shown in Figure 3D. The non-Gaussian nature of the joint probability density stems mostly from the uncertainty in  $\tau$  as shown in Figures 2A-2D and indicated by the non-zero values for the skewness and kurtosis obtained in these simulations (Table 2). All four of the marginal probability densities of  $\tau$  have tails which are skewed to the right, i.e. positive skewness. In the simulations for  $ef = 19.1$  and  $nf = 0.70$  and 0.35, the upper bound of these probability densities is defined by the prior density. The 0.95 credibility intervals for  $\tau$  in these two simulations extends from 0.14 to 11.4 ns and from 2.3 to 11.7 ns. These findings suggest that under these experimental conditions the data contain only minimal information about the molecule's fluorescent lifetime.

## Discussion

Our approach provides an exact probability model of the photon arrival times observed with background noise and makes explicit the relation between the counting process, arrival time and conditional arrival time probability densities. This model avoids the need to bin data and extends the analysis provided [ ] and [ ] by making possible ML and Bayesian analyses which consider the background noise in the estimation of the excited species lifetime and energy emission. Both procedures measure the uncertainty in these quantities based on data from the photons collected from single molecule in a single experiment.

Our ML analysis gives explicit formulae for the change in precision of the lifetime estimates as a function of the signal intensity, background noise and detection interval. ML analysis of experimental data provides the most efficient use of experimental information and as such, is the preferred approach provided the ML computations are numerically tractable the experiment is highly informative about the model parameters relative to the known prior to the experiment. Inferences about uncertainty in model parameters can be made from ML theory as long as the appropriate asymptomatic assumptions are satisfied. Contrary to the suggestion by Tellinghuisen et al., our results show that ML estimation for single molecule lifetime and energy emission and is highly tractable procedure which can be carried out efficiently using a quasi-Newton's methods. Hall and Selinger showed that when the observation interval is finite the large sample properties of the ML estimate of  $\tau$  and  $A$  are not of order  $1$  but of order  $A$ . Our ML analysis extends their result by showing that the result also hold when the data consist of a Poisson signal plus Poisson background noise model observed in finite time.

While the ML and Bayesian procedures will give similar parameter and uncertainty estimates when the expected signal intensity is large, our simulation study shows that when the number of detected photons is small, the Bayesian procedures



provide a more reliable uncertainty assessment of uncertainty. The Bayesian procedure makes it possible to combine prior information about the most probable range to  $\tau$  and  $A$  prior to the experiment with the information on these parameters summarized in the likelihood. In particular, the Bayesian procedure trades the large sample assumptions required by the ML error analysis for the more realistic one that the fluorescent lifetime and energy emission--where the latter is implicitly defined by  $\tau$  and  $A$ --lie within given intervals. As we have shown, this interval may be specified based on the known properties of the experimental paradigm. The Bayesian procedure suggests therefore, a preferred alternative when the expected signal intensity is low and as a consequence, the large sample assumptions needed to justify the ML analysis cannot be satisfied.

While either the Bayesian or ML procedures works well for analyzing fluorescence emissions, the Bayesian methods provide more realistic measures of uncertainty in the fluorescent lifetimes for any combination of background noise and fluorescence emission. The Bayesian methods should provide more realistic assessments of uncertainty in fluorescent lifetime estimates in current single molecule flow stream experiments where the expected fluorescence emission can be in the low to medium range. Both the ML and Bayesian algorithms can be automated for real-time applications.

**Acknowledgment.** This research was supported by Robert Wood Johnson Foundation Grants, 19122 and 23397, and NASA Grant NAGW 4061. The author is grateful to Herman Chernoff, Apostoles Doukas, Richard Keller and Joel Tellinghuisen for helpful discussions.

## Appendix

To derive the algorithm we note that the conditional cumulative distribution function (CDF) of  $t_i$  given  $t_{i-1}$  is

$$F(t_i|t_{i-1}) = 1 - \exp\left\{-\int_{t_{i-1}}^{t_i} \lambda(u) + q(u) du\right\} \quad (\text{A.1})$$

Equation (1) ensures that  $F(t_i|t_{i-1})$  is a proper CDF defined on  $(t_{i-1}, \infty)$ . Sequential application of the inverse CDF algorithm for Monte Carlo sampling gives the following algorithm:

STEP 0: Set  $t_0 = 0$ .

STEP 1: Given  $t_{i-1}$  draw  $r_i$  a uniform random number on  $(0,1)$ .

STEP 2: Compute  $F^{-1}(r_i) = t'$  from (A.1).

STEP 3: If  $t' < T$  then set  $t_i = t', t_{i-1} = t_i$  and go to STEP 1, otherwise stop.

The inversion in STEP 2 is computed numerically.

## References and Notes

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TABLE 1.

## MAXIMUM LIKELIHOOD ESTIMATION SUMMARY:

FLUORESCENT LIFETIME					ENERGY EMISSION		
ef	nf	MEAN	SD	CVx100%	MEAN	SD	CVx100%
19.1	0.70	2.86	1.66	58.03	16.60	7.77	46.81
19.1	0.35	4.66	2.10	44.97	19.60	5.49	27.99
19.1	0.00	3.22	0.88	27.44	22.10	4.71	21.32
191	0.70	4.10	0.85	20.82	207.25	25.02	12.07
191	0.35	4.58	0.70	15.29	221.25	17.98	8.12
191	0.00	3.74	0.42	11.22	182.75	13.55	7.41
952	0.70	4.76	0.63	13.25	876.00	55.79	6.37
952	0.35	4.04	0.28	6.88	964.00	38.19	3.96
952	0.00	4.04	0.21	5.31	908.00	30.13	3.30
3812	0.70	4.32	0.12	2.87	3950.00	63.50	1.61
3812	0.35	4.32	0.13	2.93	3880.00	65.78	1.62
3812	0.00	4.24	0.11	2.68	3870.00	62.19	1.61

TABLE 2.  
BAYESIAN ANALYSIS SUMMARY: FLUORESCENT LIFETIMES

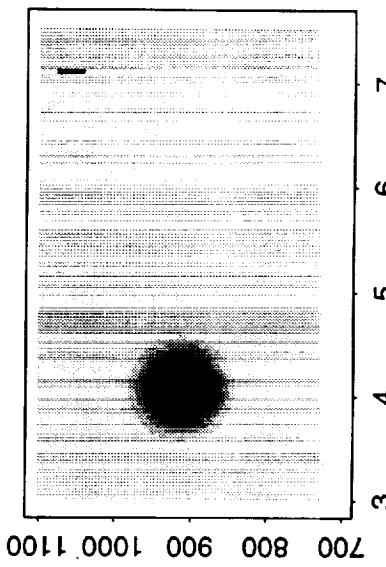
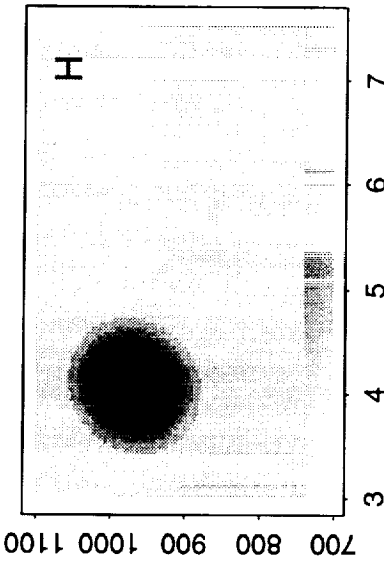
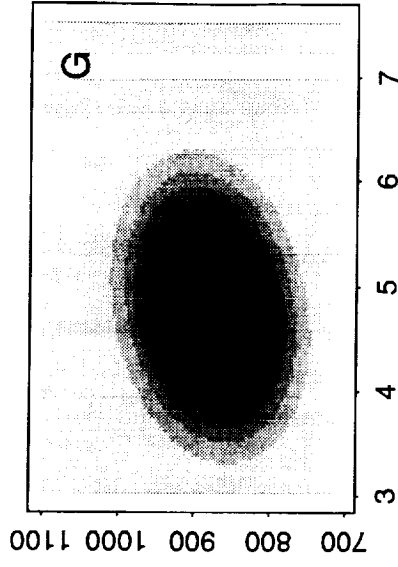
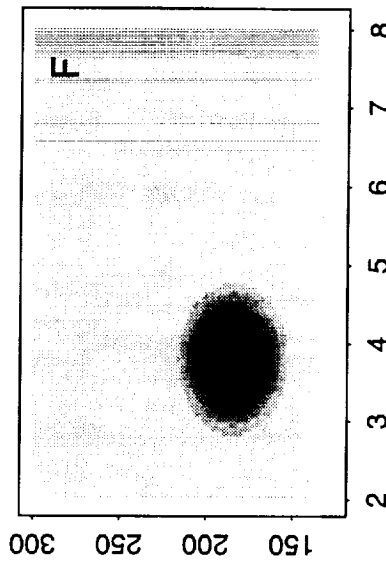
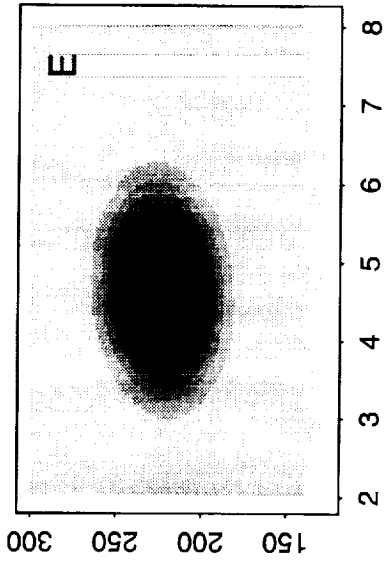
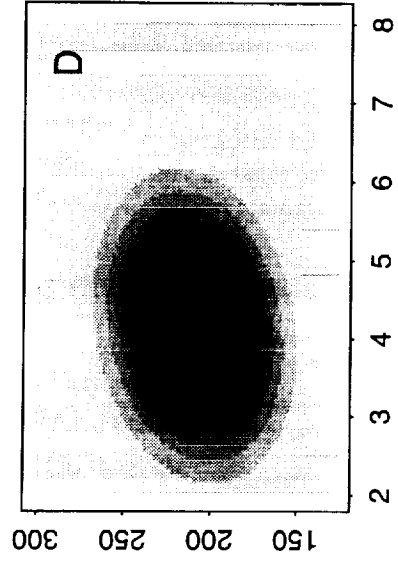
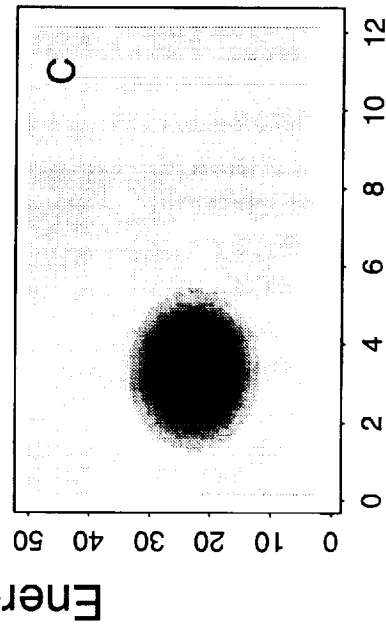
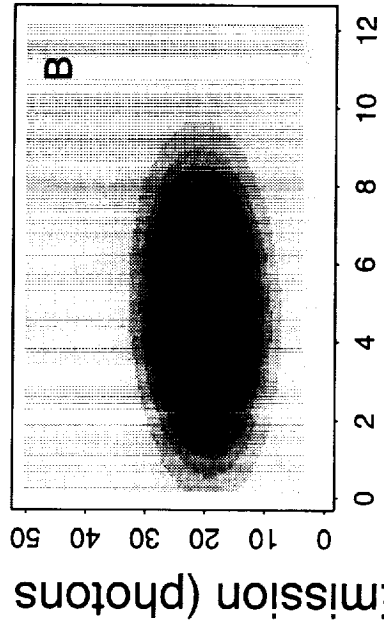
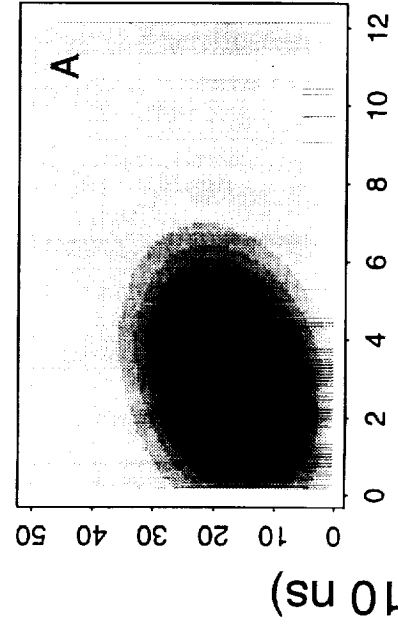
ef	nf	MODE	MEDIAN	MEAN	SD	SKEW <sup>†</sup>	KURT <sup>††</sup>	CVx100%
19.1	0.70	3.22	5.21	5.61	3.02	0.365	-0.891	53.79
19.1	0.35	4.78	6.44	6.68	2.61	0.259	-0.932	39.07
19.1	0.00	3.22	3.99	4.44	1.87	1.494	2.315	42.07
191	0.70	4.16	4.47	4.59	1.04	0.670	0.209	22.71
191	0.35	4.58	4.78	4.85	0.81	0.693	0.547	16.66
191	0.00	3.74	3.88	3.90	0.47	0.705	1.047	12.09
952	0.70	4.76	4.92	4.96	0.69	0.524	0.259	13.87
952	0.35	4.04	4.07	4.07	0.29	0.335	0.236	7.00
952	0.00	4.04	4.10	4.09	0.22	0.302	0.186	5.43
3812	0.70	4.32	4.35	4.35	0.24	0.244	0.125	5.53
3812	0.35	4.32	4.33	4.32	0.16	0.175	0.064	3.66
3812	0.00	4.24	4.25	4.24	0.11	0.149	0.045	2.69

BAYESIAN ANALYSIS SUMMARY: ENERGY EMISSION

ef	nf	MODE	MEDIAN	MEAN	SD	SKEW <sup>†</sup>	KURT <sup>††</sup>	CVx100%
19.1	0.70	17.60	18.71	18.84	7.82	0.302	-0.035	41.51
19.1	0.35	19.60	20.53	20.61	5.56	0.357	0.184	27.00
19.1	0.00	22.10	22.92	23.00	4.80	0.416	0.253	20.85
191	0.70	207.25	209.70	209.19	24.93	0.083	-0.053	11.92
191	0.35	223.00	223.77	223.23	18.03	0.108	0.006	8.08
191	0.00	182.75	183.54	183.00	13.53	0.148	0.033	7.39
952	0.70	876.00	880.70	879.08	55.58	0.048	-0.052	6.32
952	0.35	964.00	967.55	965.86	38.13	0.038	-0.042	3.95
952	0.00	908.00	910.67	909.00	30.15	0.066	0.007	3.32
3812	0.70	3950.00	3955.88	3951.25	112.47	0.021	-0.005	2.85
3812	0.35	3880.00	3890.03	3885.37	76.87	0.026	0.001	1.98
3812	0.00	3870.00	3877.66	3873.00	62.23	0.032	0.002	1.61

<sup>†</sup>The skewness of a probability density of a random variable  $X$  is the normalized third moment defined as  $E(X - \mu)^3 / \sigma^3$  where  $\mu$  is the mean and  $\sigma^2$  is the variance.

<sup>††</sup>The kurtosis of a probability density of a random variable  $X$  is the normalized fourth moment defined as  $E(X - \mu)^4 / \sigma^4 - 3$ .



Fluorescent Lifetime (ns)

